(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 283 942**
A2

(12) # EUROPEAN PATENT APPLICATION

(21) Application number: 88104294.9

(51) Int. Cl.4: **C12N 5/00**

(22) Date of filing: 18.03.88

(30) Priority: 24.03.87 US 29577

(43) Date of publication of application:
28.09.88 Bulletin 88/39

(84) Designated Contracting States:
DE FR GB IT

(71) Applicant: **W.R. GRACE & CO.**
Grace Plaza 1114 Avenue of the Americas
New York New York 10036(US)

(72) Inventor: **Wolfe, Richard A.**
10208 Shaker Drive
Columbia, Md. 21046(US)

(74) Representative: **UEXKÜLL & STOLBERG**
Patentanwälte
Beselerstrasse 4
D-2000 Hamburg 52(DE)

(54) **Basal nutrient medium for cell culture.**

(57) This defined basal nutrient medium is very effective in both high and low density culture of a wide variety of cell lines and cell types. The medium may be used for serum-free culture or supplemented with low levels of serum. The medium contains a buffer system formulated for air equilibration.

EP 0 283 942 A2

BASAL NUTRIENT MEDIUM FOR CELL CULTURE

BACKGROUND OF THE INVENTION

This invention relates generally to a medium for the in vitro culture of mammalian cells. More specifically, the invention is a defined basal nutrient medium for serum-free culture or for culture when supplemented with low levels of serum. For serum-free culture, defined proteins are added to the basal nutrient medium. The medium is very effective when used in either high or low density culture of a wide variety of cell lines and cell types.

For in vitro culture, a medium must, of course, supply all essential nutrients for the cells: vitamins, amino acids, lipids, nucleic acid precursors, carbohydrates, trace elements, and bulk ions. Historically, basal nutrient media were designed to support cell growth only after being supplemented with a biological extract, e.g., serum or embryo extracts. Serum, in particular, proved to be an effective supplement, presumably because it contains the necessary growth-and multiplication-promoting factors in physiologically acceptable concentrations. Examples of basal nutrient media of this type are Eagle's basal medium (BME), the composition of which is recited in U.S. 3,450,598 (Welsh et al.), and Dulbecco's Modified Eagle's (DME) medium, the composition of which is recited in Table II of Ham et al., "Media and Growth Requirements," Methods of Enzymology, (1978). DME medium, which contains relatively high concentrations of the essential amino acids and sugars, is representative of the commercially available media formulated for the mass culture of cells with serum supplementation.

With growing sophistication in cell culture techniques, factors present in serum or other biological extracts have been identified. It is now possible to grow mammalian cells in a serum-free environment, by supplementing a basal nutrient medium with defined proteins necessary for cell growth and multiplication. For example, Ham's F12 medium was formulated for clonal protein-free growth. F12, the composition of which is given in Table II of Ham et al., supra, contains low concentrations of the essential amino acids and sugars, and includes lipids, nucleic acid derivatives, vitamins and nonessential amino acids.

It is now generally accepted that a readily obtainable and sufficiently complex basal nutrient medium for mass culture of cells in low serum concentrations can be fabricated by mixing DME and F12 media. Such mixtures, when supplemented with the appropriate protein factors, can also support the serum-free growth of many cell types. Barnes et al., "Methods for Growth of Cultured Cells in Serum-Free Medium," Analytical Biochem., Vol. 102, pp. 255-70 (1980), describes examples of both approaches.

Several commercially available nutrient media are based on mixtures of DME, F12 and/or other media such as those listed in Table II of Ham et al., supra. However, simple mixtures of existing commercial media are by no means optimal for culturing all cell lines and medium preparations therefore have been targeted largely to particular cell lines or cell types. Wolfe et al., "Continuous Culture of Rat C6 Glioma in Serum-Free Medium," J. Cell Biol., Vol. 87, pp. 434-41 (1980), teaches the use of a 3:1 DME-to-F12 mixture, supplemented with trace elements, and further supplemented with the following defined proteins: insulin, transferrin, fibroblast growth factor, linoleic acid complexed to fatty acid-free bovine serum albumin, and serum-spreading factor (vitronectin).

With the increasing use of cultured mammalian cells to produce biologicals (e.g., monoclonal antibodies and genetically engineered proteins), there is an increasing demand for chemically defined, serum-free media. Purification of the desired cellular product is greatly complicated by the presence of serum, which may contain at least several hundred different proteins. It is therefore desired to reduce the protein content of the culture medium to a few defined compounds from which the monoclonal antibody or other cellular product can be separated more readily.

In addition to necessary nutrients and protein factors, the medium must have a means for controlling pH levels. Most typically, pH control relies on a bicarbonate/carbon dioxide buffer system, which requires carbon dioxide regulators as well as incubators which supply a constant level of carbon dioxide to the culture. The buffering capacity of the system can be expanded by the inclusion of a biocompatible organic buffer, such as alpha-glycerolphosphate or HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid). Alternatives to the cumbersome bicarbonate/carbon dioxide buffer system have been proposed, but have not received significant acceptance in the field. See, for example, Leibovitz, "The Growth and Maintenance of Tissue-Cell Cultures in Free Gas Exchange with the Atmosphere," Am. J. Hygiene, Vol. 78, pp. 173-80 (1963), disclosing a medium (L-15) which uses free base amino acids and substitutes D(+)galactose, sodium pyruvate and DL-alpha-alanine for glucose. It is taught that the L-15 medium can be used in free gas exchange with the atomosphere.

SUMMARY OF THE INVENTION

The basal nutrient medium of the present invention is designed for high or low density mammalian cell culture when supplemented either with defined protein factors or very low levels of serum. The medium contains a buffer system which utilizes air equilibration.

The primary objective of this invention is to provide a chemically defined medium which is advantageous for high density culture of mammalian cells and which supports such culture in the absence of serum or in the presence of very low serum concentrations. One important intended benefit is reduction of the concentrations of growth inhibitors that are present in serum. In addition, by providing a culture medium with low levels of exogenous protein, recovery and purification of the desired cell product will be facilitated.

A more specific object is to design a medium having a sodium/potassium ratio and a total osmolarity compatible with high levels of immunoglobulin production by hybridoma cells. It is also intended that the medium be free of polypeptides which co-purify with immunoglobulins. It is a goal of this invention to markedly improve the purity of the cellular product recovered from the culture.

It is an additional goal to provide a cell culture medium particularly well suited for use in hollow fiber bioreactors. To achieve this goal, it is intended that the medium formulation be capable of avoiding or reducing changes in osmolarity sufficient to have a deleterious effect on the cultured cells.

It is a further object to design a medium having nutrients at levels which are suitable for high cell densities, but which are not inhibitory for low density culture. It is intended to eliminate the need for media changes when going from low to high density culture conditions, as well as to reduce or eliminate the need for "weaning" cells from serum-supplemented to serum-free media.

Still another object of the invention is to design a basal nutrient medium which is suitable for the culture of a wide variety of cell types and sources. It is intended that this medium be compatible with the clonal growth of human cells.

Yet another important object is to provide a medium with a buffer system that is formulated for equilibration with air, rather than with mixtures of air and carbon dioxide.

BRIEF DESCRIPTION OF THE FIGURE

FIGURE 1 is a listing of the components of the medium of this invention, including the molecular weight and the concentration of each component.

DETAILED DESCRIPTION OF THE INVENTION

The basal nutrient medium described herein is a completely new formulation of nutrients and other components which is suitable for both high and low cell density culture. The medium comprises appropriate levels of essential and non-essential amino acids and amino acid derivatives, bulk ions and trace elements, buffers, vitamins, coenzymes, carbohydrates and derivatives, nucleic acid derivatives, and lipids to function as an all-purpose nutrient medium for *in vitro* mammalian cell culture. The basal medium is designed to be supplemented either with defined proteins or with low levels of serum or other biological extracts. The buffer system of this medium is specially formulated to allow for air-equilibrated pH control.

It is typical for nutrient media to be individually designed for specific uses or for the growth of particular cell lines or cell types. Prior art formulations for high cell densities frequently have been found to contain high concentrations of certain components that are grossly inhibitory for low cell density culture. In addition, many prior media have been specifically formulated for growth of one cell line and contain components whose concentrations have been optimized for that cell line only. No prior art medium or combination of media contains all of the components of the presently described media, nor are individual components used in the same concentrations as in any prior art media.

The medium described herein is an all-purpose basal nutrient medium. It has been demonstrated to effectively support both low and high density cell culture. It has been demonstrated to supply the nutrients needed by a variety of cell lines and types. The medium is particularly well-suited for production of monoclonal antibodies in a variety of production modes, such as hollow fiber bioreactors, fermentors, spinner flasks and roller bottles. Where the medium is supplemented with defined proteins (i.e., hormones and growth factors) or with low levels of serum, high purity cell products, e.g. monoclonal antibodies, are readily recoverable.

Most of the major nutrients and other factors essential for cell growth are known and have been used

previously in many combinations and permutations. The concentrations of the components, however, have been newly formulated for the nutrient medium of this invention. The components have not merely been optimized for one particular cell line or set of production conditions, as is commonly done in this industry. Rather, the components have been optimized as an interrelated set of growth factors and enhancers in an all-purpose culture medium. In addition, the pH control system differs dramatically from prior art media.

The components described herein and listed in FIGURE 1 are given in the physical and ionization states common in the art of media preparation. However, other physical and/or ionization states may be used, if desired. The concentration of any of the components, with the exception of HEPES and sodium hydroxide, may be varied from that listed in FIGURE 1 by as much as a factor of two as long as the osmolarity, pH and sodium-to-potassium ratio are within the ranges described herein. The HEPES concentration can range from about 15.0 to about 28.0 mM. The quantity of NaOH used is a function of the pH selected.

Bulk Ions and Trace Elements -Bulk ions are necessary for cell growth and for maintenance of membrane potentials and osmotic balance. They also play co-factor roles in enzymatic reactions. Sodium, potassium, calcium, magnesium, chloride, phosphate, and sulphate all perform important functions in normal cell metabolism. The specific sodium-to-potassium ratio in the medium, important in regulating transmembrane potential, is discussed further below. Bicarbonate or carbon dioxide is also necessary, and must be provided in the culture medium for low density cell culture. In high density cell culture, the cells themselves may generate sufficient levels, without the need for exogenous bicarbonate and carbon dioxide. Trace inorganic elements (iron, zinc, selenium, silicon, vanadium, copper, nickel and molybdenum) are necessary for the function of many enzymes (e.g., Se^{++} in glutathione reductase). Trace inorganic elements also can directly modulate transmembrane signaling events (e.g., vanadate modulation of insulin responsiveness). The specific compounds listed in FIGURE 1 are commonly used in media preparations and are preferred here because the indicated hydration states are advantageous for the stability of the powdered form of the medium of this invention. Substitutions may be made by those of ordinary skill in the art.

Amino Acids -The following essential amino acids are included in this medium: L-arginine (L-Arg), L-cysteine (L-Cys), L-glutamine (L-Gln), L-histidine (L-His), L-hydroxyproline (L-Hydroxy-Pro), L-isoleucine (L-Ile), L-leucine (L-Leu), L-lysine (L-Lys), L-methionine (L-Met), L-phenylalanine (L-Phe), L-threonine (L-Thr), L-tryptophan (L-Trp), L-tyrosine (L-Tyr), and L-valine (L-Val). In addition, the following non-essential amino acids are included: L-alanine (L-Ala), L-asparagine (L-Asn), L-aspartic acid (L-Asp), L-glutamic acid (L-Glu), glycine (Gly), L-proline (L-Pro) and L-serine (L-Ser). In addition, the amino acid derivatives glutathione and putrescine are present in the medium of this invention. Again, the forms listed in FIGURE 1 are preferred, particularly for the preparation of a powdered medium that will dissolve readily. For preparation of a liquid medium, alternative forms of these amino acids may be selected.

Vitamins/Coenzymes -A number of water soluble vitamins and co-enzymes are known to aid cell culture. Biotin, pantothenic acid, folic acid, folinic acid, niacinamide (nicotinamide), p-aminobenzoic acid, pyridoxal, pyridoxine, riboflavin, thiamine and vitamin B_{12} are utilized in this medium.

Carbohydrates -Glucose, pyruvate and glutamine are utilized as the energy and carbon sources in the present medium. Pyruvate is provided as sodium pyruvate. It may be desired for process control to alter the components used by the cells as an energy source. For example, the glucose may be replaced by galactose or fructose, and the glutamine concentration varied.

Nucleic Acid Derivatives -Adenine and hypoxanthine are provided as sources of purines. Thymidine is provided as a source of pyrimidines.

Lipids -The formulation of this invention includes the following lipids, lipid precursors and lipid derivatives: choline, ethanolamine, i-inositol, linoleic acid and lipoic acid. Additional lipids and other derivatives such as methyl lineolate may be added or substituted as required for particular cell types. Ethanolamine is a major component in the membrane phospholipid biosynthetic pathway.

Buffers -The buffer system of the nutrient medium described herein is unique. This system offers the operator the ease and flexibility of using air equilibration for pH control. This is an important aspect of the present invention, since the medium is primarily intended for serum-free or very low serum concentration culture. It has been found that when the serum concentration is reduced, the levels of bicarbonate normally suitable for pH control in equilibrium with 10% carbon dioxide/air become inhibitory. The present buffer system also offers an alternative to the burdensome adjustment of carbon dioxide concentrations which previously have been required for maintaining the pH within physiologically compatible ranges.

The buffer system utilizes sodium bicarbonate, HEPES (n-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), sodium hydroxide and carbon dioxide. The small quantities of carbon dioxide required for cellular metabolism in low density cultures are provided in the medium of this invention via equilibration of atmospheric carbon dioxide and the HCO_3^- present in the medium. For high density cultures, sufficient

carbon dioxide is produced via normal cell metabolism. Particularly in the case of hollow fiber bioreactors, which have very densely packed beds of functioning cells, large quantities of carbon dioxide are generated. If the buffering system is based on carbon dioxide/bicarbonate equilibrium, the environment can rapidly become too acidic for the cells to function at optimal levels. The air-equilibrated buffer system in the present medium eliminates this problem.

The need for using the pH indicator phenol red is eliminated in the medium of this invention, since the buffer system of this medium will maintain the pH within physiological ranges under common culture conditions in an air-equilibrated system. This is extremely advantageous in terms of purifying the desired cellular product, since phenol red binds to proteins, changing their chromatographic behavior. In addition, phenol red may affect cellular biosynthesis and metabolism. Elimination of phenol red is therefore significant in terms of reducing the required purification steps and increasing recoverable product.

The medium may be formulated at about pH 7.0 to about pH 7.4 at 37°C. Formulation at a higher pH, for example, at about pH 8.0, may be employed as a process control strategy for continuously fed bioreactors to neutralize the lactic acid produced by the cultured cells, instead of adding additional base as a process control strategy. When the medium is to be used in a hollow fiber bioreactor, formulation at about pH 7.35 (37°C) is preferred. A pH of 7.2 (37°C) is preferred for other uses.

Osmolarity -The sodium/potassium ratio and total osmolarity of the medium have been adjusted for compatibility with high levels of murine immunoglobulin production. The preferred sodium-to-potassium ratio is about 30, but may range from about 25 to about 35. The osmolarity of the medium is low, about 285 to about 315 mosm, preferably about 295 to about 305 mosm.

The medium described herein is particularly well suited for the production of monoclonal antibodies in hollow fiber bioreactors, fermentors, spinner flasks and roller bottles. The high levels of gas exchange routinely employed in these types of culture are compatible with the present formulation. The osmolarity of the medium has been kept low to allow for some rise during culture, while still maintaining the osmolarity within ranges suitable for maintaining healthy, productive cells. For use in hollow fiber reactors, the medium preferably is reconstituted at about 295 mosm. In addition, biocompatible reducing agents, such as glutathione have been included in the medium to compensate for potential oxidative complications arising from these high levels of gas exchange.

The formulation for the basal nutrient medium of this invention is listed in FIGURE 1. Quantities of the components are given in molarity as well as concentration. The formulation of FIGURE 1 is the preferred embodiment of this invention. The quantity of each component may be varied by a factor of 2, that is, the quantity of each component may vary from about 50% to about 200% of the quantity listed in FIGURE 1. The concentrations for each component have been selected on the basis of the mechanism by which it enters the cell, i.e., active or passive transport, and the concentrations required to achieve sufficient transport for the desired level of biological activity.

The hydration state of the individual components and the prepared basal nutrient medium may be varied according to convenience. The hydration states given herein are those which are commonly used in the art of media preparation. However, as a practical matter, it is preferred to have the prepared medium be as dry as possible.

The basal nutrient medium as described above may be formulated and packaged as a dry or concentrated preparation for reconstitution prior to use. In the preferred embodiment of this invention, the medium is prepared as a dry powder, comprising the first sixty components listed in FIGURE 1. The remaining two components are then added when the dry medium is reconstituted. Reconstitution may be done just prior to use. Alternatively, the medium may be reconstituted and packaged. The shelf life of this medium as a dry powder stored at about 4°C is at least several years. The liquid medium, either as prepared or as reconstituted from the dry powder is less stable, but when stored at about 4°C is stable for about two months or more.

Reconstitution may be performed by adding concentrated stocks of bicarbonate, base or other of the medium components, so long as the relative concentrations described above and indicated in FIGURE 1 are present. If those components are added as solids, reconstitution is accomplished by the addition of sterile, de-ionized tissue culture grade water. The medium is sterilized prior to use. A protocol for reconstituting the powdered medium is detailed in Example I.

Additional antioxidants, reducing agents, vitamins, carbohydrates, amino acids and/or derivatives, nucleic acids and/or derivatives, and proteins may be added prior to, during or after reconstitution. The principal added components will be proteins and/or other hormones.

As stated above, the basal nutrient medium of this invention is designed to be supplemented either with low levels of serum or with defined proteins. The medium will support cell growth and metabolism when supplemented with amounts of serum appropriate for the particular cell line being cultured. However, it has

been demonstrated that considerably lower levels of serum are required to supplement the medium of this invention than are typically used with the prior art media. This formulation may be used serum-free or with very low levels of serum, preferably less than about one percent by volume, although higher levels may be used if desired.

The medium described herein can be used for serum-free cell culture when supplemented with a minimum number of defined proteins. The precise protein supplement will depend on the needs of the cells being cultured. The preferred protein supplement for murine hybridomas consists of insulin, albumin and iron-saturated transferrin. Insulin may be present in concentrations of about 1.0 to about 10.0 µgm/ml, preferably about 5.0 µgm/ml. Albumin may be present in concentrations of about 10.0 to about 1000.0 µgm/ml, preferably about 50.0 µgm/ml. Iron-saturated transferrin may be present in concentrations of about 0.1 to about 25.0 µgm/ml, preferably about 1.0 µgm/ml. Supplementing the basal nutrient medium with these three proteins has been found to be excellent for both high and low density cell culture. Additional proteins may be added if desired.

The examples which follow are given for illustrative purposes and are not meant to limit the invention described herein. The following abbreviations have been used throughout in describing the invention:

BSA - bovine serum albumin
°C - degree(s) Centigrade
cm^2 -cubic centimeter(s)
DME - Dulbecco's Modified Eagle's
TF - transferrin
gm - gram(s)
L - liter(s)
M - molar
mM - millimolar
mg - milligram(s)
min - minute(s)
ml - milliliter
mosm - milliosmolality (µmol/Kg)
MW - molecular weight
N - normal
nm - nanometer(s)
osm - osmolality (mmol/Kg)
µ -micro-
PBS - phosphate buffered saline
% - percent
rpm - revolution(s) per minute
v - volume
wt - weight

EXAMPLE I

(Preparation of Medium)

Powdered Medium -The medium was prepared by mixing the first sixty components listed in FIGURE 1 (that is, all components with the exception of $NaHCO_3$ and NaOH) in the quantities listed in FIGURE 1. The ingredients are milled to form a dry powder.

Stock Solutions -Biocarbonate/base ($NaHCO_3$/NaOH) stock solutions were prepared as follows:

(1) For pH 7.2 (37°C), the stock was prepared by adding 17.922 gm $NaHCO_3$ to 711.2 ml of a 1.00 N solution of NaOH. The volume was then adjusted to one liter.

(2) For pH 7.35 (37°C)) hollow fiber bioreactor medium, the stock was prepared by adding 17.922 gm $NaHCO_3$ to 950.0 ml of a 1.00 N solution of NaOH. The volume was then adjusted to one liter.

Albumin/Transferrin stock solution was prepared using bovine serum albumin (BSA) (Miles Diagnostics, Pentex grade, $2^{\times}$ recrystallized) and bovine iron-saturated transferrin (TF) (Miles Diagnostics) at 1000-fold the concentrations of the reagents in the final preparation. For 100.0 ml stock, 5.0 gm BSA, 0.1 gm TF, and 10.0 ml of $10^{\times}$ PBS (Dulbecco's Ca^{++}-, Mg^{++}-free) (GIBCO/BRL). The stock was filter sterilized and stored at 4°C.

Insulin stock solution was prepared by dissolving bovine insulin (INS) (Sigma I5500) at 1000-fold the concentration in the final preparation. For 100.0 ml stock, 500.0 mg INS were dissolved in a solution of 0.05 M HCl in PBS (using 1.0N HCl and 10× PBS (Dulbecco's Ca^{++}-, Mg^{++}-free) (GIBCO/BRL)). The stock was filter sterilized and stored at 4°C.

Reconstitution and Protein Supplementation of Powdered Medium -Six liters of tissue culture grade water were placed in a 10.0 liter vessel, to which a 195.7 gm quantity of the powdered medium (a ten liter-equivalent package) was added. The package was rinsed twice with 100.0 ml aliquots of water. Next, 150.0 ml (15.0 ml/L of medium) of the bicarbonate/base stock solution was added to the vessel. The appropriate bicarbonate/base stock solution was selected, depending on the use to which the medium would be put. The sides of the vessel were rinsed with 630.0 ml water to insure that all the powder dissolved. A 10.0 ml (1.0 ml/L of medium) aliquot of each of the albumin/transferrin stock and the insulin stock were added. Three liters of water were added to bring the volume to 10.0 L.

The pH of the standard reconstituted medium was determined (at 37°C) to be 7.18 ± 0.03 with a blood gas analyzer (Corning). The pH of the bioreactor reconstituted medium was determined to be 7.35 ± .03 (37°C). The osmolarity was determined to be 295 ± 5.0 mosm by vapor pressure osmometry (Wescor).

The reconstituted medium was filter sterilized using a Masterflex(TM) pump (#25 head) (Cole-Parmer) at approximately 500.0 ml/min. The solution was passed through a Milli-stack GS (TM) filter (Millipore MSG-SO5C22) into sterile glass and polycarbonate carboys.

The reconstituted medium was tested to verify sterility and ability to promote cell proliferation. A 10.0 ml aliquot of medium was sterilely placed in a tissue culture flask (T-75) to which one million CRL 1606 murine hybridoma cells (obtained from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852) were added. A 100 µL aliquot then was diluted with 10.0 ml PBS and the cell concentration determined using a Coulter Counter (TM) particle counter (Coulter Electronics). The flask was tightly clasped and incubated at 37°C for 24 hours. At least 200,000 cells/ml were observed, indicating the ability of the medium to support the culture.

The bottled medium was left at room temperature overnight to verify sterility. No cloudiness or other evidence of microbial contamination was observed. The medium was then stored at 4°C.

EXAMPLE II

This example compares cell growth and monoclonal antibody production in the serum-free medium of Example I versus serum-supplemented DME (Dulbecco's Modified Eagle's) medium. Better cell growth and antibody production were seen with the medium of this invention.

An aliquot of cells of the murine hybridoma line CRL 1606 was inoculated as shown in Table II into the reconstituted medium of Example I (pH 7.2). This was designated Culture IIA. Another aliquot of cells was inoculated into DME medium (GIBCO/BRL) supplemented with 10% (v/v) fetal bovine serum (GIBCO/BRL). This was designated Culture IIB. The media and cells were placed in polystyrene roller bottles (Corning, 490 cm^2). The bottle containing Culture IIA was tightly sealed. The bottle containing Culture IIB was gassed with 10% carbon dioxide/air prior to sealing. Both bottles were placed in an incubator at 37°C on a roller apparatus at about 1.0 rpm.

Aliquots of each culture were removed daily and the cell concentrations were determined with a Coulter Counter (TM) particle counter (Coulter Electronics). Cell viability was determined by the trypan blue dye exclusion assay (Sigma Chemical Co.) The results are shown in Table I.

The cells were removed from each daily aliquot by centrifugation. The conditioned medium supernatant from each aliquot was stored at 4°C until termination of the experiment. The antibody (anti-fibronectin IgG) present in each aliquot was determined by ELISA analysis.

TABLE I

	Viable Cells (billions/L)		Antibody (mg/L)	
Hour	IIA	IIB	IIA	IIB
0	0.021±.001	0.022±.001	--	--
25	0.040±.001	0.021±.001	3.2	2.2
47	0.098±.001	0.059±.001	8.1	4.5
71	0.310±.017	0.185±.005	16.6	11.5
97	1.200±.016	0.740±.026	80.3	100.0
120	2.300±.030	2.000±.028	262.9	149.6
144	0.910±.018	1.500±.010	307.8	238.6
169	--	--	1000.0	420.0

EXAMPLE III

This example compares cell growth and antibody production in the medium of this invention versus another serum-free medium, both supplemented with the same defined proteins. As in Example II, CRL 1606 cells were inoculated as shown in Table II (Hour 0) into roller bottles containing either the medium of this invention (Culture IIIA) or medium prepared from commercially available preparations (Culture IIIB). Culture IIIA was prepared by inoculating the medium of Example I (pH 7.2). Culture IIIB was prepared by mixing 3 parts DME medium (GIBCO/BRL) with 1 part F12 medium (GIBCO/BRL), 25 mM HEPES (Sigma Chemical Co.), 0.02 mM ethanolamine (Sigma Chemical Co.) and 3.0 mM sodium bicarbonate (Sigma Chemical Co.), and supplementing with:

50.0 µgm/ml bovine serum albumin (ICN Biologicals),
1.0 µgm/ml iron-saturated human transferrin (ICN Biologicals), and
5.0 µgm/ml bovine insulin (Sigma Chemical Co.).

The pH of both preparations was adjusted with sodium hydroxide until the value approached 7.2 at 37°C according to a Corning blood-gas analyzer. The osmolarity of both formulations was adjusted to approximately 295 mosm according to a Wescor vapor-pressure osmometer. The procedures of Example I were repeated. The results are shown in Table II.

TABLE II

Hour	Viable Cells (billions/L) IIIA	Viable Cells (billions/L) IIIB	Antibody (mg/L) IIIA	Antibody (mg/L) IIIB
0	0.050±.001	0.049±.001	--	--
21	0.094±.004	0.086±.004	9.4	12.5
46	0.360±.009	0.290±.006	20.1	21.0
71	1.100±.003	0.700±.010	73.6	72.5
93	2.000±.032	1.400±.014	209.7	132.0
117	0.630±.012	1.100±.004	452.3	136.2
140	--	0.440±.019	457.0	231.0
165	--	--	792.0	165.0

EXAMPLE IV

This example compares the purity of monoclonal antibody produced in cultures based on media of this invention versus that produced in cultures based on serum-supplemented media.

Two roller bottles were inoculated with $2.1\pm0.1 \times 10^4$ CRL 1606 cells per milliliter of medium. One bottle (Culture IVA) contained the medium of Example I (pH 7.2). The other bottle (Culture IVB) contained DME medium supplemented with 10% (v/v) fetal bovine serum. Samples of the conditioned medium were prepared as described in Example II after one and four days of culture. The proteins contained in the samples were then examined by high performance size exclusion chromatography (HPSEC, Zorbax (TM) (E.I. dupont de Nemours Co.) 0.75 × 25 mm column isocratic 0.3M sodium chloride/0.05m sodium phosphate; pH 7.0; 0.25 ml/min).

Table III presents the relative amounts of materials migrating with retention times similar to standards of highly purified BSA (bovine serum albumin) or IgG (immunoglobulin G). Absorbance at 280 nm was measured, and the integrated peak areas determined. In addition, it is shown that the monoclonal antibody produced in Culture IVB was undetectable amongst the bovine IgG and albumin (3.2±.35 mg/ml total IgG and albumin) present by virtue of the 10% fetal bovine serum supplement.

TABLE III

Sample	Peak Area Units IgG (Rf ≃ 16.7)	Peak Area Units BSA (Rf ≃ 17.7)
IVA, Day 1	Baseline	5,770 (Rf=17.63)
IVA, Day 4	15,647 (Rf=16.75)	5,470 (Rf=17.95)
IVB, Day 1	Buried	686,528 (Rf=17.63)
IVB, Day 4	Buried	641,592 (Rf=17.92)

At 280 nm, the absorbance of a 1.0 gm/ml solution of IgG is 1.715 times that of a solution of 1.0 mg/ml BSA. Thus, the data in Table III suggests that the Culture IVA, Day 4 sample contained approximately 170

µgm/ml IgG and that the monoclonal antibody was about 63% pure:

$$\frac{[IgG]}{[BSA] + [IgG]} = \frac{1.668 \times [BSA]}{[BSA] + (1.668 \times [BSA])}$$

$$= \frac{1.668 \times [BSA]}{(1+1.668) \times [BSA]}$$

$$= \frac{1.668}{1 + 1.668}$$

$$= 0.63 = 63\%$$

where:

$$[IgG] = \frac{A_{280}IgG \quad 1.715}{A_{280}BSA} \times [BSA]$$

$$= 1.668 \times [BSA]$$

In addition, it appears that highly purified IgG can be obtained after only a simple size exclusion chromatographic step. Crude conditioned medium of both higher product titer and greater product purity can easily be obtained by culturing the cells for a few more days. Under the conditions of this example, the cells in Culture IVA had only reached a density of 1,000,000 cells/ml by Day 4, which is less than half the saturation density.

EXAMPLE V

This example demonstrates the utility of this medium for reviving cells from cryopreservation. The example also demonstrates that the described medium can support the proliferation of cells which previously have been cultured in the presence of high serum concentrations, without the need for prior adaptation ("weaning") procedures.

Cells of the HB127 cell line (SP2/0 Ag14) were obtained from ATCC. This cell line had always been grown in serum-supplemented media. This SP2/0 murine hybridoma line was derived from a different parental fusion partner than the CRL 1606 line used in Examples I-IV. These two cell lines together are representative of most of the monoclonal antibody producing cells commonly used in the industry. The examples demonstrate that both grow better in the medium of this invention than in serum-supplemented DME medium.

The cells were thawed and divided into two equal portions. The portions were incubated overnight at 37°C in 5.0 ml of either the reconstituted medium of Example I (pH 7.2), (Culture VA) or in DME medium supplemented with 10% serum (Culture VB). On day 2, the medium in each culture was exchanged via centrifugation with a fresh preparation. The cultures were returned to the incubator. On day 5, the cells in each culture were counted. In Culture VA, 687,000 cells/ml were observed, versus only 251,000 cells/ml in Culture VB. Therefore, even this cell line which had always been grown in serum-supplemented media grew significantly better in the serum-free medium of this invention, without any intermediate weaning cultures being necessary.

EXAMPLE VI

This example demonstrates the suitability of this medium for the culture of anchorage dependent cell lines in the presence of low concentrations of serum. Cell lines were examined from two different species and two different tissues.

For this experiment, LLC-PK1 (porcine kidney) cells (ATCC number CL101) and CPAE (bovine endothelial) cells (ATCC number CCL 209) were obtained from ATCC. The cell inoculum for each cell line was prepared by treating stock cultures with 5.0 ml of a solution of Trypsin/EDTA (ethylenediamine

tetraacetate) (GIBCO/BRL) and suspending the single cells in the reconstituted culture medium of Example I (pH 7.2). The cells of each cell line then were added to triplicate T-25 flasks containing the medium of this invention, reconstituted from the powder of Example I by the reconstitution procedure described (pH 7.2) but without adding the protein stock solutions of Example I. The medium was supplemented with fetal bovine serum at the concentrations shown in Table IV. The flasks were sealed and placed in a 37°C incubator.

After five days of culture, the medium was removed from each flask, and the cultures were treated with Trypsin/EDTA solution as above. After the cells had detached from the surface of the flask, they were resuspended in a phosphate buffered saline solution. The cell number was determined with a Coulter Counter (TM) particle counter (Coulter Electronics). The results are shown in Table IV.

TABLE IV

Cell Line	Serum	Cells per Flask	
		Initial	Day 5
CL 101	1%	9.5×10^4	$11.4 \pm .4 \times 10^5$
CCL 209	2%	1.0×10^4	$3.3 \pm .2 \times 10^4$

EXAMPLE VII

The medium of this invention was used for the production of monoclonal antibodies in an Amicon Vitafiber II-P30 (TM) (Amicon Division of W. R. Grace & Co.) hollow fiber bioreactor (nominal ultrafiltrative cut-off of 30,000 MW). The system was constructed to permit exchange of fresh medium with the medium that was continuously recirculated through the hollow fiber cartridge. The rate of this exchange was increased as the cartridge filled with cultured cells. The final exchange rate (for the filled cartridge) was approximately 2.0 L/day, and 81.0 L of medium were used during the entire bioreactor run.

Following sterilization and assembly, the hollow fiber cartridge and bioreactor system were sequentially flushed with tissue culture grade water and with the reconstituted medium of Example I (pH 7.2). The medium-contacting surfaces of the system were coated with protein via incubation with the medium of this invention, reconstituted from the powder of Example I by the reconstitution procedure described (pH 7.2) with the following protein supplement: 1.0 mg/ml BSA, 1.0 µgm/ml TF, and 5.0 µgm/ml INS. The sterility of the system was verified by visual observation of medium clarity after operation for three days with this medium.

The cartridge was then inoculated with three hundred million CRL 1606 cells. Daily samples were taken and measured for pH. Each time the pH dropped below 6.8, the medium feed rate was increased, up to a maximum of 2.0 L/day. When that rate was reached, the medium was switched to an identical medium reconstituted at pH 7.35.

Samples were taken from the cell space as indicated in Table V, and the monoclonal antibody product (MAB) was characterized and quantified by HPSEC as in Example IV. In most of the samples, the purity of the MAB was greater than 95%. The cumulative production of MAB by this hollow fiber bioreactor over 49 days of operation with the medium of this invention is shown in Table V.

Table V

Total Days of Operation	Cumulative Production (gm)
7	0.006
10	0.022
14	0.370
18	1.441
21	2.202
24	2.570
28	2.852
36	3.458
39	3.847
44	4.299
48	4.662

Similar results were obtained using other hollow fiber bioreactors constructed with other fibers having 10,000 and 30,000 MW nominal ultrafiltrative cut-offs.

The principles, preferred embodiments and modes of operation of the present invention have been described in the foregoing specification. The invention which is intended to be protected herein, however, is not to be construed as limited to the particular forms disclosed, since these are to be regarded as illustrative rather than restrictive. Variations and changes may be made by those skilled in the art without departing from the spirit of the invention.

Claims

1. A basal nutrient medium for in vitro mammalian cell culture, comprising the components listed in FIGURE 1.

2. The nutrient medium of Claim 1 in which each of said components is present in a quantity of from about fifty to about two hundred percent of the quantity listed in FIGURE 1.

3. The nutrient medium of Claim 1 which has an osmolarity of about 285 to about 315 milli-olsmols, preferably about 295 to about 305 milli-osmols.

4. The nutrient medium of Claim 1 which has a pH (37°C) of about 7.0 to about 8.0, preferably of about 7.2 to about 7.35.

5. The nutrient medium of Claim 1 which has a sodium-to-potassium ratio of about 25 to 35, preferably of about 30.

6. The nutrient medium of Claim 1 which is supplemented with defined proteins.

7. The nutrient medium of Claim 6 in which said proteins comprise albumin, iron-saturated transferrin and insulin, whereby preferably albumin is present in concentrations of about 10.0 to about 1000 micrograms per milliliter, iron-saturated transferrin is present in concentrations of about 0.1 to about 25.0 micrograms per milliliter, and insulin is present in concentrations of about 1.0 to about 10.0 micrograms per milliliter.

8. The nutrient medium of Claim 1 which is supplemented with serum or another biological extract, preferably in an amount as up to about one percent of the medium by volume.

9. A dry basal nutrient medium preparation comprising the first sixty components listed in FIGURE 1.

10. The dry medium preparation of Claim 9 in which each of said components is present in a quantity of from about fifty to about two hundred percent of the quantity listed in FIGURE 1.

FIGURE 1

COMPONENT	MW	M	mg/L
Bulk Ions & Trace Elements			
$CaCl_2 \cdot 2H_2O$	147.02	$1x10^{-3}$	147.02
$CuSO_4 \cdot 5H_2O$	249.68	$3x10^{-9}$	0.000749
$FeSO_4 \cdot 7H_2O$	278.02	$1x10^{-6}$	0.278
$Fe(NO_3)_3 \cdot 9H_2O$	404.02	$2x10^{-7}$	0.0808
KCl	74.55	$4x10^{-3}$	298.2
$MgSO_4 \cdot 7H_2O$	246.38	$8x10^{-4}$	197.1
NaCl	58.44	$1.05x10^{-1}$	6136.2
$Na_2HPO_4 \cdot 7H_2O$	268.1	$3x10^{-4}$	80.43
$NaH_2PO_4 \cdot 2H_2O$	156.01	$6x10^{-4}$	93.606
$Na_2SeO_3 \cdot 5H_2O$	263.01	$3x10^{-8}$	0.00789
$Na_2SiO_3 \cdot 9H_2O$	284.2	$1x10^{-2}$	2.842
$(NH_4)6Mo_7O_{24} \cdot 4H_2O$	1235.9	$3x10^{-9}$	0.00371
NH_4VO_3	116.99	$5x10^{-10}$	0.0000585
$NiSO_4 \cdot 6H_2O$	262.80	$3x10^{-10}$	0.0000788
$ZnSO_4 \cdot 7H_2O$	287.54	$8x10^{-7}$	0.23
Essential Amino Acids			
L-Arg	210.7	$8x10^{-4}$	168.56
L-Cys HCl . H_2O	175.6	$3x10^{-4}$	52.68
L-Gln	146.1	$5x10^{-3}$	730.5
L-His HCl . H_2O	209.7	$2x10^{-4}$	41.94
L-Hydroxy-Pro	131.13	$1x10^{-4}$	13.113
L-Ile	131.2	$6x10^{-4}$	78.72
L-Leu	131.2	$6x10^{-4}$	78.72
L-Lys HCl	182.7	$8x10^{-4}$	146.16
L-Met	149.2	$1x10^{-3}$	149.2
L-Phe	165.2	$3x10^{-4}$	49.56
L-Thr	119.1	$6x10^{-4}$	71.46
L-Trp	204.2	$6x10^{-5}$	12.252
L-Tyr (diNa^+) $2H_2O$	237.2	$3x10^{-4}$	71.16
L-Val	117.2	$6x10^{-4}$	70.32

FIGURE I (Cont'd)

COMPONENT	MW	M	mg/L
Nonessential Amino Acids			
L-Ala	89.09	$2x10^{-5}$	1.782
L-Asn . H_2O	150.1	$3x10^{-4}$	45.03
L-Asp	133.1	$2x10^{-5}$	2.662
L-Glu	147.1	$2x10^{-5}$	2.942
Gly	75.07	$3x10^{-5}$	2.252
L-Pro	115.1	$2x10^{-4}$	23.02
L-Ser	105.1	$3x10^{-4}$	31.53
Amino Acid Derivatives			
Glutathione	307.3	$1x10^{-6}$	0.307
Putrescine 2HCl	161.1	$3x10^{-7}$	0.048
Water Soluble Vitamins and Co-Enzyme			
Biotin	244.3	$3x10^{-8}$	0.007
D-Ca pantothenate	238.3	$2x10^{-5}$	4.766
Folic acid	441.41	$6x10^{-6}$	2.648
Folinic acid (Ca^{+}) $5H_2O$	601.6	$1x10^{-6}$	0.602
Niacinamide (Nicotinamide)	122.1	$3x10^{-5}$	3.663
p-Aminobenzoic acid	137.14	$3x10^{-6}$	0.411
Pyridoxal HCl	203.6	$1x10^{-5}$	2.036
Pyridoxine HCl	205.6	$3x10^{-7}$	0.062
Riboflavin	376.4	$8x10^{-7}$	0.301
Thiamine HCl	337.0	$9x10^{-6}$	3.036
Vitamin B12	1355.4	$3x10^{-7}$	0.407
Carbohydrates and Derivatives			
D-Glucose	180.16	$2x10^{-2}$	3603.2
Na Pyruvate	110.0	$1x10^{-3}$	110.0
Nucleic Acid Derivatives			
Adenine	135.13	$1x10^{-6}$	0.135
Hypoxanthine (Na^{+})	146.1	$7x10^{-6}$	1.0227
Thymidine HCl	337.3	$1x10^{-5}$	3.373

FIGURE I (cont'd)

COMPONENT	MW	M	mg/L
Lipids and Derivatives			
Choline chloride	139.63	$1x10^{-4}$	13.96
Ethanolamine HCl	97.55	$2x10^{-5}$	1.951
i-Inositol	180.2	$1x10^{-4}$	18.02
Linoleic acid	280.4	$1x10^{-7}$	0.028
Lipoic acid	206.3	$2x10^{-7}$	0.041
Buffers			
HEPES	238.3	$2.5x10^{-2}$	5957.5
NaOH	40.01	$1.23x10^{-2}$	492.12
$NaHCO_3$	84.01	$3x10^{-3}$	252.03
mM sodium (total)		123.1	
mM potassium (total)		4.0	
sodium:potassium		30.7	
total osmolarity (mosm)		302.7	